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14. ABSTRACT

We have shown that infusions of opioid-like hibernation factors can provide ischemic protection in vivo and in vitro. In a mouse cerebral ischemia model, we showed a marked reduction in cerebral infarct volume and behavioral deficits when Deltophins-A, D and Dermorphin-H were infused 1 hr after middle cerebral artery occlusion. Using mice deficient in either neuronal (nNOS), inducible (iNOS) or endothelial (eNOS) nitric oxide synthase, we showed that only endothelial NOS plays a key role in cerebral ischemic protection. We found that Deltorphin-D inhibited nitric oxide (NO) release in a dose dependent manner when mouse N9 microglial cells were activated by lipopolysaccharide (LPS) and interferon-gamma, giving further evidence that the neuroprotective effect of these delta opioids may include their ability to retard or block the release of NO and reactive oxygen species which occur in ischemic processes such as hemorrhagic shock, myocardial infarction, and stroke. Most recently, we showed that Deltorphin-D increased blood pressure and enhances 6 hr survival of rats undergoing hemorrhagic shock without concomitant fluid resuscitation.

15. SUBJECT TERMS
Opioids, Deltorphins, Dermorphins, Ischemic injury

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FINAL REPORT

GRANT #: N00014-01-1-0494

INSTITUTION: University of Kentucky College of Medicine

GRANT TITLE: Neuroprotective Effects of Opioid-Like Hibernation Factors in Cerebral Ischemia

AWARD PERIOD: 1 March 2001- 31 March 2004

OBJECTIVE: To determine the mechanism(s) by which opioid-like hibernation factors provide cerebral ischemic protection and increase blood pressure and survival following hemorrhage shock in clinically relevant animal models.

APPROACH:

1. **Cerebral Ischemia Studies:** To determine if nitric oxide (NO) mediates the neuroprotective effects of highly specific delta opioids (Deltorphin A, Deltorphin-D and Demorphin-H). Wild type and nitric oxide deficient (NOS) all on a C57B16 background were subjected to one hr occlusion of the middle cerebral artery (MCA) and 24 hr of reperfusion. Brain infarct volume and behavioral deficits were measured in control mice and in mice which were deficient in inducible nitric oxide (iNOS), neuronal nitric oxide (nNOS) endothelial cell nitric oxide (eNOS).

ACCOMPLISHMENTS:

Injection of saline into the NOS deficient mice resulted in infarct volumes previously demonstrated in the literature, where iNOS and nNOS deficient animals have reduced damage due to the detrimental effects of NO synthase in the brain. In contrast deficient animals showed an increase in infarct volume indicating a protective effect of eNOS in vascular function in cerebral ischemia. Injection of the Deltorphin-A, Deltorphin-D and Dermorphin-H resulted in a significant protection of the brain in wild-type animals and infusion gave rise to similar effects in the NOS deficient mice as seen in the saline injected study. Since there was no enhancement of protection in the NOS deficient mice, these data suggest that some of the protective effects of Deltorphin-D are mediated through nitric oxide.

CONCLUSIONS:

Our data indicate that opioids, which are highly specific for the *delta*$_2$ opioid receptor subtype, can provide profound cerebral ischemia protection (i.e., significantly decreased infarct volume vs. controls) when infused in the tail vein of mice one hour after occlusion of the middle cerebral artery. Utilizing gene inactivation mice indicates that *delta* opioid ischemic neuro protection may involve a mechanism requiring activation of endothelial cell NO synthase.

APPROACH:

2. In Vitro Cell Culture Assay To Monitor The Nitric Oxide (NO) Inhibitory Activity of Delta Opioid-Like Hibernation Factors.

To develop a rapid *in vitro* cell culture assay by which we could monitor NO inhibitory activity of *delta* opioid-like hibernation factors alone or in combination with curcumin, a potent antioxidant. This was accomplished utilizing the Griess Assay (Oshima et, al, Carcinogenesis 12: 1217-1220, 1991)

ACCOMPLISHMENTS:

Four different opioid peptides were used to test their effect on inhibition of NO production in an *in-vitro* cell culture assay. Delt-D, DPDPE (D-Pen ^{2,5})-Enkephalin, a $delta_1$ selective opioid. DADLE (D-Ala^2-Leu^5-Enkephalin), a non-specific *delta* opioid and Dermorphin-H a *mu* selective opioid were used. In addition Curcumin, an antioxidant reactive oxygen species (ROS) scavenger and known NO synthase inhibitor was also used as a positive control. Among the opioids only Delt-D inhibited NO production in a concentration-dependent manner. Delt-D at a concentration of 1 mM inhibited NO production by 95% when compared to activated control cells. Curcumin at a concentration of 50 μM also inhibited NO production by 95% when compared to activated control cells whereas even 2.5 mM concentration, DADLE has no effect on NO release by activated cells. In activated cells that were treated with 1 mM DPDPE there was a slight increase in NO production when compared to activated control cells.

CONCLUSIONS:

Our data indicates that only Delt-D, a *delta* opioid which is highly specific for the $delta_2$ receptor subtype, inhibits NO release in an activated microglial cell line in a concentration dependent manner. DPDPE, which is a *delta* opioid highly specific for the $delta_1$ receptor subtype does not inhibit NO release but in fact enhances NO release compared to controls. These data indicated that opioids binding the $delta_2$ receptor subtype may initiate cellular mechanisms resulting in ischemic protection.

APPROACH:

3. Hemorrhagic Shock Studies: Rats weighing 300-350 g had catheters placed in the femoral artery (for hemorrhage), tail artery for blood pressure (BP) measurement and the tail vein (for administration of opioids) controls received saline or opioids without hemorrhage. BP and 6 hr survival were monitored.

Moderate Hemorrhage Protocol: For the moderate hemorrhage studies (5.5 ml hemorrhage volume prior animals received saline or Delt-D to hemorrhage without fluid resuscitation and post-treated animals received saline or Delt-D 2 mg/kg following hemorrhage without fluid resuscitation. BP, blood loss and rectal temp, at beginning and end of hemorrhage were determined. The effect of Delt-D infusions on the expression of Ubiquitin B and C (UBB and UBC) was determined. Heat Shock Protein (HSP-70), and inducible Nitric Oxide Synthase (iNOS) mRNA transcripts in heart, leg and brain were determined after 2 hr.

ACCOMPLISHEMENTS: Moderate Hemorrhage Protocol: Preinfusions of Delt-D did not significantly effect BP while 2 mg/kg post hemorrhage infusions without

resuscitation fluid significantly increased BP compared to controls and decreased core temp by 4.5 °F compared to controls. Delt-D infusions increased iNOS and HSP70 mRNA in heart and leg in non-hemorrhaged controls and UBB in brain of non-hemorrhaged controls. Pre-treated Delt-D animals had elevated brain iNOS and HSP70 mRNA and post-hemorrhage Delt-D treated animals had elevated UBC mRNA in heart and brain and HSP70 mRNA in leg tissue.

APPROACH:

Severe Hemorrhage Protocol: For the severe hemorrhage protocol (9.0 – 11.0 ml hemorrhage volume representing 53-61% of total blood volume), rats were infused with either 3.0 mg/kg of a highly specific *mu* opioid ZGI-06, (n-11), in 1.0ml PBS, or a Delt-D variant (ZGI-07 n=11) and ischemic tolerance (ie BP and 6 hr survival) was monitored. Controls (n=6) were infused with 1.0 ml PBS.

ACCOMPLISHEMENTS:

Severe Hemorrhage Protocol: Six hr survival was 33% for controls (n=2), 60% for ZGI-06 (n=6) and 72% for ZGI-07 (n=8) BP increased within 30-45 seconds after infusion of ZGI-06 by 29.5± 13.0 mmHg vs. control (p=0.01) and 38.8 ± 18.5 mmHg for ZGI-07 vs. control, (p=0.002).

CONCLUSIONS:

Moderate Hemorrhage Protocol:

Pre-hemorrhage infusions of Deltorphin-D do not significantly alter BP compared to saline controls. Delt-D at 2 mg/kg increase blood pressure and decreased core temperature vs. saline controls during the 1st hour of hemorrhage without concomitant fluid resuscitation.

Severe Hemorrhage Protocol:

Highly specific *delta* opioid(ZGI-07) and a highly specific *mu* opioid (ZGI-06) increased BP and enhanced 6 hr. survival in rat undergoing profound hemorrhage (50% blood loss or>) without concomitant fluid resuscitation.

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